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Thanks,

Alana M. Harris, Ph.D.  
Biotech. Patent Examiner  
Art Unit 1642  
CM-1/ 8A17  
(703) 306-5880

# Integrin Activation Suppresses Etoposide-induced DNA Strand Breakage in Cultured Murine Tumor-derived Endothelial Cells<sup>1</sup>

Dale G. Hoyt,<sup>2</sup> James M. Rusnak, Robert J. Mannix, Ruth A. Modzelewski, Candace S. Johnson, and John S. Lazo

Departments of Pharmacology [D. G. H., J. M. R., R. J. M., C. S. J., J. S. L.] and Otolaryngology [R. A. M., C. S. J.], University of Pittsburgh School of Medicine, and the University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15261

## Abstract

Tumor endothelium is critical for solid tumor growth and is a potential site for anticancer drug action. Within 2 h, etoposide caused marked DNA strand breakage in xenograft tumor-derived endothelial cells (TDECs). Etoposide-induced DNA breakage was inhibited by culturing TDECs on gelatin, type IV collagen, laminin, fibronectin, and the integrin ligand hexapeptide, GRGDSP, but not the inactive peptide, GRADSP. It was also inhibited when TDECs were on surfaces coated with antibodies to  $\alpha_5$ ,  $\beta_1$ , or  $\beta_3$  integrin subunits and by clustering integrins with soluble antibodies. After 8 h with etoposide, TDECs detached from the monolayer, and 50-kb DNA fragments were seen. Fibronectin inhibited both processes. Thus, integrins are survival factors for TDEC that inhibit the genotoxicity of etoposide and may influence the sensitivity of tumors to drugs.

## Introduction

Endothelial cells of tumors are potentially important targets of chemotherapy since tumor development depends on vascularization (1). The sensitivity of endothelial cells to anticancer agents may be influenced by endogenous regulators of survival, such as fibroblast growth factor and tumor necrosis factor (2, 3).

Cell survival is also regulated by the extracellular matrix via integrin adhesion receptors. Thus, apoptosis caused by preventing attachment of endothelial cells is inhibited by activation of  $\alpha_5\beta_1$  integrin (fibronectin-selective) and  $\alpha_v\beta_3$  integrin (vitronectin-selective; Refs. 4-6). Integrins also protect epithelial cells from death due to growth factor withdrawal (7-9), and extracellular matrix protects endothelial cells from radiation injury (10). Previously, we found that lung endothelial cells cultured on gelatin, basement membrane proteins (type IV collagen, laminin, and fibronectin), an integrin-ligand hexapeptide, or antibodies to integrin subunits were protected from DNA strand breakage caused by bacterial endotoxin (LPS)<sup>3</sup> (11, 12).

The topoisomerase II inhibitor, etoposide, is an important anticancer drug that causes apoptosis in other cell types (13). The effect of etoposide on tumor endothelium has not been determined. Furthermore, it is not known whether protection of endothelial cells by integrin activation is specific for LPS or whether endothelial cells from different vascular sources are subject to protection. Hence, we have examined the effect of integrin activation on the toxicity of etoposide and LPS in TDECs.

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<sup>2</sup> To whom requests for reprints should be addressed, at Department of Pharmacology, E1347 Biomedical Science Tower, University of Pittsburgh, Pittsburgh, PA 15261. Phone: (412) 383-7783; Fax: (412) 648-1945.

<sup>3</sup> The abbreviations used are: LPS, lipopolysaccharide; TDEC, tumor-derived endothelial cell; ISBE, *in situ* break extension; ISNT, *in situ* nick translation.

## Materials and Methods

**Materials.** Etoposide, *Escherichia coli* DNA polymerase I, and H33258 were from Boehringer-Mannheim (Indianapolis, IN). Terminal deoxynucleotidyl transferase and fluorescein-12-dUTP were obtained from Stratagene (La Jolla, CA). PBS, LPS (*E. coli* serotype 0111:B4), gelatin (type A from porcine skin; 300 Bloom), goat antirat IgG, and rabbit antihamster IgG were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse type IV collagen and laminin were purchased from Collaborative Research (Bedford, MA). Bovine plasma fibronectin, GRGDSP, and GRADSP, were obtained from Calbiochem (La Jolla, CA). Rat antimouse  $\alpha_5$  integrin, rat antimouse  $\beta_1$  integrin and hamster antimouse  $\beta_3$  integrin antibodies were obtained from PharMingen (San Diego, CA). Cell culture media and reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT).

**TDECs.** TDECs were isolated from RIF-1 tumors in C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) and characterized as described previously (14). Briefly, tumors were removed, dissociated and labeled with monoclonal antibody against angiotensin-converting enzyme, and sorted by flow cytometry. Angiotensin-converting enzyme-positive cells were further purified based on uptake and metabolism of acetylated low density lipoprotein. Cultures were not used after 10 passages and were maintained in endothelial cell growth medium that consisted of DMEM enriched with 20% Sarcoma 180-conditioned medium, 10% fetal bovine serum, 5 units/ml heparin, 1.34  $\mu$ M L-glutamine, Eagle's basal medium with vitamins, and 50  $\mu$ g/ml of endothelial cell growth supplement (Collaborative Research, Lexington, MA).

**Coating of Culture Slides.** Slides were coated with extracellular matrix proteins as described previously (12). Slides were incubated 4 h at 37°C with 2% gelatin or 10  $\mu$ g/cm<sup>2</sup> mouse type IV collagen, mouse laminin, or bovine plasma fibronectin. The hexapeptides, GRGDSP and GRADSP, were also coated on slides at 10  $\mu$ g/cm<sup>2</sup>. After incubation, the solutions were removed, slides were rinsed with PBS (137 mM sodium chloride, 8 mM dibasic sodium phosphate, 3 mM potassium chloride, and 1.5 mM monobasic potassium phosphate, pH 7.4), air dried, and stored at 4°C (12). As described previously, anti-integrin antibodies were used to activate specific integrins (5, 12). Slides were coated with rat antimouse  $\alpha_5$  integrin, rat antimouse  $\beta_1$  integrin, and hamster antimouse  $\beta_3$  integrin. Some slides were precoated with an appropriate isotype-specific secondary antibody to enhance clustering of integrins (12). Other surfaces were left untreated or coated with secondary antibodies alone to serve as controls. Cells (50,000) were placed in each chamber with DMEM/20% fetal bovine serum (medium). The cells were treated with medium or with medium containing etoposide or LPS 24 h later.

**Addition of Anti-Integrin Antibodies to Medium.** The effect of integrin clustering with anti-integrin antibodies added to medium was also tested as described previously (12). TDEC were first placed in each slide chamber. After 24 h, the medium was removed, and TDEC were incubated with medium or medium containing 1  $\mu$ g/ml of a specific anti-integrin antibody for 1 h at 4°C. When used, secondary antibodies were added at a final concentration of 2  $\mu$ g/ml. The temperature was then raised to 37°C. After 1 h, medium or medium containing etoposide (0.1 volume) was added to give a final concentration of 0 or 10  $\mu$ M etoposide. DNA strand breaks were assayed 2 h later.

**ISBE and ISNT to Quantify DNA Strand Breaks.** Cells were washed three times with PBS at 4°C after treatment with LPS or etoposide. Remaining attached cells were fixed with 1% formaldehyde/PBS and made permeable with 70% ethanol. DNA breaks were labeled with terminal deoxynucleotidyl transferase and fluorescein-12-dUTP, as described previously (11). DNA

breaks were also labeled by ISNT as described by Gorczyca *et al.* (15) with the substitution of fluorescein-12-dUTP. Cells were incubated at 37°C for 90 min with 2.5 mM MgCl<sub>2</sub>, 50 mM Tris (pH 7.8), 10 mM  $\beta$ -mercaptoethanol, 10  $\mu$ g/ml BSA, 16  $\mu$ M each of dGTP, dATP, and dCTP, 16  $\mu$ M fluorescein-12-dUTP, and 2 units *E. coli* DNA polymerase I/ml. Labeling was stopped by rinsing with PBS.

For both ISBE and ISNT, DNA breaks were indicated by nuclear fluorescence intensity, obtained from digital image analysis with a Meridian ACAS 570c laser scanning confocal microscope (Okemos, MI). Nuclei were outlined graphically, and the fluorescence intensity (signal/area) was recorded. Cells (100 to 350) were analyzed for each medium-treated and etoposide-treated group. The mean difference between the etoposide-treated and medium-treated groups  $\pm$  SE of the difference is presented. Data were analyzed by Student's *t* test (16). Our previous studies document the expected equivalent results obtained from statistical analysis of fluorescence intensity values or from quantal data generated by defining cells as positive or negative relative to an arbitrary fluorescence intensity (12).

**Cell Detachment and DNA Fragmentation.** Cell detachment was determined by collecting detached cells and separately releasing attached cells with 0.05% trypsin/2 mM EDTA. The DNA content of each fraction was determined by H33258 fluorescence assay. The amount of DNA in the detached fraction was divided by the total amount of DNA (11). Internucleosomal and higher order DNA fragmentation were determined by normal agarose and field inversion gel electrophoresis, as described previously (11).

## Results and Discussion

**DNA Strand Breaks.** Within 2 h after exposure of TDECs to etoposide, DNA strand breakage was obvious (Fig. 1A). Maximum strand breakage was seen with 10 and 30  $\mu$ M etoposide. The apparent decrease in DNA breakage with 100  $\mu$ M etoposide could be due to detachment of severely injured cells after 2 h. Time- and concentration-dependent DNA breakage was also seen with LPS (Fig. 1B), recapitulating our previous results with pulmonary endothelial cells (12). We also found that etoposide-induced DNA breaks were removed when TDECs were incubated 2 h in drug-free medium. Culturing TDECs on fibronectin suppressed early DNA strand breakage by both etoposide and LPS.

Endothelial cells normally reside on basement membrane containing type IV collagen, laminin, and fibronectin. We cultured TDECs on slides coated with these matrix proteins or GRGDSP or GRADSP peptides, as described previously (12). We found that type IV collagen, laminin, fibronectin, and gelatin protected TDECs from etoposide (Figs. 1 and 2). Furthermore, a coating of GRGDSP, but not the integrin-inactive GRADSP peptide, reduced DNA strand breakage. These data strongly suggested that activation of integrins by RGD-containing matrices was responsible for inhibition of DNA strand breakage in response to etoposide.

$\alpha_5$ ,  $\beta_1$ , and  $\beta_3$  integrins have been implicated in the regulation of endothelial cell apoptosis, and activation of integrins with specific antibodies has been used to prevent detachment-induced apoptosis (5, 6). Thus, TDECs were plated on slides coated with anti- $\alpha_5$ , - $\beta_1$ , and - $\beta_3$  integrin antibodies. Secondary antibodies alone did not prevent DNA strand breaks caused by 10  $\mu$ M etoposide. Anti-integrin antibodies inhibited etoposide-induced DNA breaks alone and in the sandwich configuration with the secondary antibodies (Fig. 3A). Integrins were also clustered by the addition of anti-integrin antibodies to the medium (Fig. 3B). The specific anti-integrin antibodies protected TDECs, and secondary antibodies alone did not. These results implicate integrins in suppression of the genotoxicity of etoposide.

**Cellular Detachment and DNA Fragmentation.** Etoposide (10  $\mu$ M) increased detachment of TDECs upon continuous incubation from 100  $\pm$  13.7% of control to 153  $\pm$  19.8 after 8 h and 169  $\pm$  17.9% after 24 h (mean  $\pm$  SE; *P* < 0.05 for etoposide-treated cells). Detachment was not significantly increased at 2 or 4 h (129  $\pm$  24 and 126  $\pm$  17% of control) or in TDECs cultured on

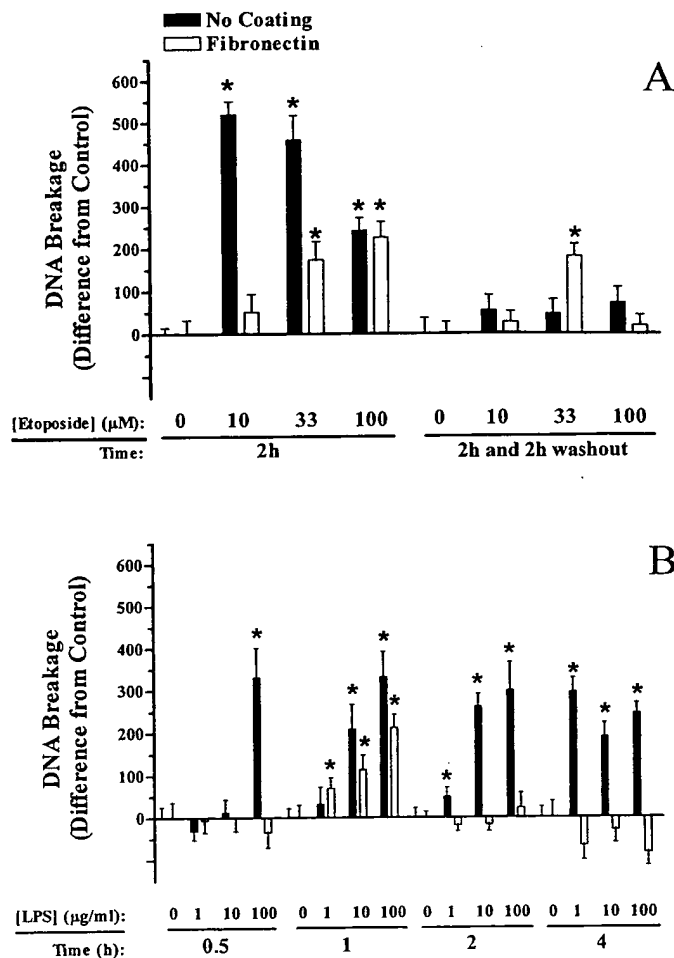


Fig. 1. Effect of fibronectin on DNA strand breakage in TDECs. In A, TDECs were placed in slides, cultured for 24 h, and treated with medium or medium containing 0, 10, 30, or 100  $\mu$ M etoposide for 2 h (2h). Cells were incubated for an additional 2 h in drug-free medium as well (2h and 2h washout). TDECs were then processed for DNA strand breakage by ISNT. Bars, SE. In B, TDECs were treated with medium containing 0, 1, 10, or 100  $\mu$ g LPS/ml for 0.5–4 h and processed for DNA strand breakage by ISBE. Images of 100–350 cells in each experimental group were analyzed for nuclear fluorescence intensity as described in "Materials and Methods." Data are the mean difference in signal intensity (drug-treated cells minus medium-treated control cells); bars, SE. \*, *P* < 0.05 for comparison with medium-treated cells.

fibronectin, where detachment as a percentage of control was 100  $\pm$  15 for medium-treated cells, 104  $\pm$  26.8 8 h after etoposide treatment, and 132  $\pm$  22.7 after 24 h (*P* > 0.05). Thus, although etoposide caused detachment that was reduced by fibronectin, it did not precede the onset of DNA strand breakage.

We extensively examined TDECs for etoposide-induced internucleosomal DNA fragmentation. No DNA laddering was seen in TDECs treated with 10–100  $\mu$ M etoposide from 2 to 24 h (data not shown). Apoptosis has been observed in other cell types, however, by us (17) and others (18), without the formation of 180-bp ladders. Thus, we characterized high molecular weight DNA fragmentation by field inversion gel electrophoresis (Fig. 4). There was a time- and concentration-dependent production of 50-kb DNA fragments that are consistent with an apoptotic process we have seen in other endothelial cells treated with LPS (11). The 50-kb DNA fragments were evident after 8 and 24 h incubation with 10 and 30  $\mu$ M etoposide. Growing TDECs on fibronectin inhibited this DNA fragmentation.

DNA strand breakage caused by etoposide is almost certainly initiated by a different mechanism than LPS; etoposide is a potent inhibitor of topoisomerase II, and there is no evidence for inhibition by LPS. *In situ* labeling tags DNA breaks containing 3'OH that are

most likely generated by a reaction of cells to the initial biochemical lesions caused by LPS and etoposide. These sites are, however, removed upon incubation in drug-free medium. The >600-kb DNA fragments caused by etoposide in MOLT-4 cells are also removed upon removal of etoposide (19). Overall, the data are consistent with the idea that apoptosis proceeds from early initiation and later commitment phases (20). Integrins are known to influence regulators of apoptotic commitment, such as bcl-2 and interleukin 1 converting enzyme. Our results further indicate that extracellular matrix inhibits the initiation of apoptosis by diverse agents. Thus, basement membrane supporting tumor endothelium could play a significant role at several levels in regulating the response of the endothelium of solid tumors to anticancer drugs.

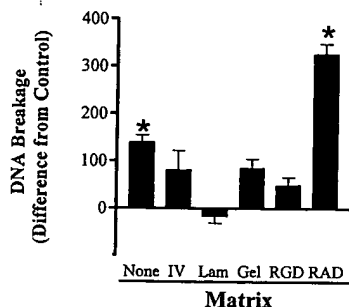


Fig. 2. Effect of specific matrices on etoposide-induced DNA strand breakage in TDECs. TDECs were placed in matrix-coated slides, cultured for 24 h, and treated with medium or medium containing 10  $\mu$ M etoposide for 2 h. Slides were coated with nothing (None), type IV collagen (IV), laminin (Lam), gelatin (Gel), GRGDSP (RGD) or GRADSP (RAD) peptide. Cells were processed for labeling by ISNT and analyzed as in Fig. 1; bars, SE.

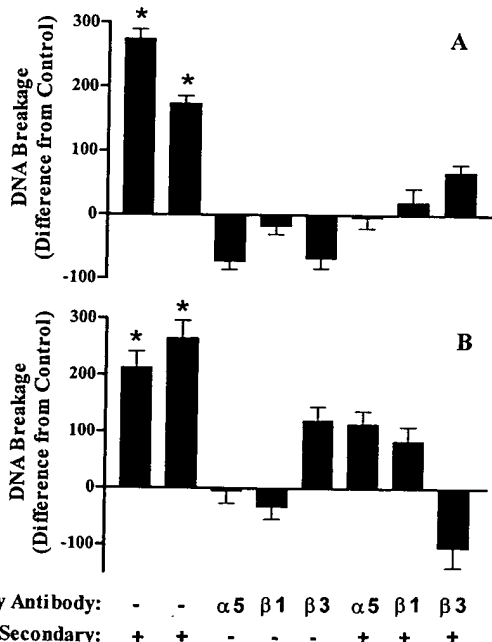


Fig. 3. Effect of anti-integrin antibodies on etoposide-induced DNA strand breakage in TDECs. In A, TDECs were incubated in slides coated without (-) or with secondary antibodies (Secondary; +), without (-) or with antibodies to murine  $\alpha 5$ ,  $\beta 1$ , or  $\beta 3$  integrins (Primary Antibody), or with anti-integrin antibodies plus secondary antibodies. After 24 h, cells were treated for 2 h with 0 or 10  $\mu$ M etoposide, processed, and analyzed as in Fig. 1A. In B, TDECs were first placed in uncoated slides for 24 h. Integrins were activated by incubating cells 1 h without or with primary antibodies (1  $\mu$ g/ml; 4°C), followed by 1 h without or with secondary antibody (2  $\mu$ g/ml; 37°C). After preincubation with antibodies, medium or concentrated etoposide was added to yield 0 or 10  $\mu$ M etoposide. After 2 h at 37°C, cells were processed for measurement of DNA strand breaks as in Fig. 1A. Data and symbols are as in Fig. 1; bars, SE.

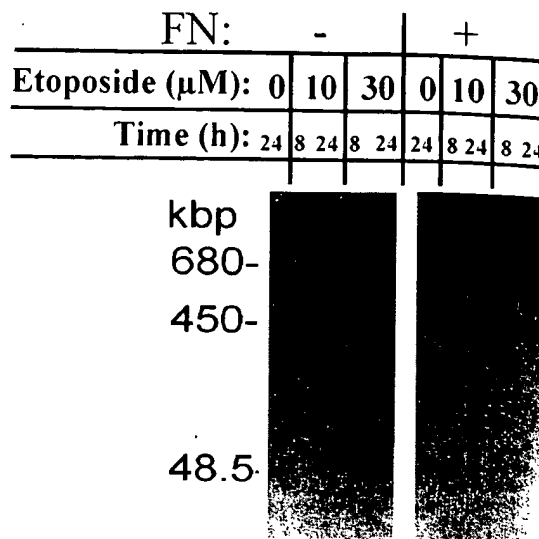


Fig. 4. Etoposide-induced high molecular weight DNA fragmentation. TDECs were plated without (-) or with (+) fibronectin (FN) and treated with 0, 10, or 30  $\mu$ M etoposide for 8 or 24 h. Cells were harvested and prepared for field inversion gel electrophoresis and staining with ethidium bromide. A negative photographic image was taken. Left, migration of the molecular weight standards in kilobases.

It is not known how integrin activation suppresses the early phase of apoptosis. It is reasonable to propose that signal transduction by integrins impinges on the nuclear response to DNA-damaging agents (21). Further studies will illuminate the effect of integrins on DNA damage and repair and on the cellular response to DNA strand breakage.

In summary, our results demonstrated that extracellular matrix proteins inhibited etoposide- and LPS-induced DNA strand breakage in TDECs. Integrins appeared to be responsible since GRGDSP peptide and anti-integrin antibodies inhibit etoposide-induced DNA strand breakage. Thus, integrin activation is a significant factor controlling the sensitivity of normal and tumor endothelium to diverse DNA-damaging agents.

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